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# Stoichiometry of a Ligand-gated Ion Channel Determined by Fluorescence Energy Transfer*

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**We have developed a method to determine the stoichiometry of subunits within an oligomeric cell surface receptor using fluorescently tagged antibodies to the individual subunits and measuring energy transfer between them. Anti-c-Myc monoclonal antibody (mAb 9-E10) derivatized with a fluorophore (europium cryptate, EuK) was used to individually label c-Myc-tagged $\alpha_1$-, $\beta_2$-, or $\gamma_2$-subunits of the hetero-oligomeric γ-aminobutyric acid ($GABA_A$) receptor in intact cells. The maximal fluorescent signal derived from the $\alpha_1$(c-Myc)$\beta_2\gamma_2$ and the $\alpha_1\beta_2$(c-Myc)$\gamma_2$ receptors was twice that obtained with $\alpha_1\beta_2\gamma_2$(c-Myc), suggesting that there are 2× α-, 2× β-, and 1× γ-subunits in a receptor monomer. This observation was extended using fluorescence energy transfer. Receptors were half-maximally saturated with EuK-anti-c-Myc mAb, and the remaining $\alpha_1$(c-Myc) subunits were labeled with excess anti-c-Myc mAb derivatized with the fluorescence energy acceptor, XL665. On exposure to laser light, energy transfer from EuK to XL665 occurred with $\alpha_1$(c-Myc)$\beta_2\gamma_2$ and $\alpha_1\beta_2$(c-Myc)$\gamma_2$, but no significant energy transfer was observed with $\alpha_1\beta_2\gamma_2$(c-Myc) receptors, indicating the absence of a second γ-subunit in a receptor monomer. We confirm that the $GABA_A$ receptor subtype, $\alpha_1\beta_2\gamma_2$, is composed of two copies each of the α- and β-subunits and one copy of the γ-subunit (*i.e.* $(\alpha_1)_2(\beta_2)_2(\gamma_2)_1$) and conclude that this method would have general applicability to other multisubunit cell surface proteins.**

Understanding the assembly and molecular interactions of proteins is a rapidly growing field in biology. Many techniques have been used to probe the interaction of proteins including, for example, immunoprecipitation, yeast two-hybrid analysis, circular dichroism, and more recently fluorescence energy transfer between two proteins in close proximity (1–3). We have used the technique of fluorescence resonance energy transfer to investigate the stoichiometric assembly of a $GABA_A$[1] receptor, a member of the major inhibitory ligand-gated ion channel family in the brain.

It is proposed that the $GABA_A$ receptors are hetero-oligomeric pentamers composed of α, β, and a third subunit type, γ, δ, or ϵ. This is based on an analogy with other ligand-gated ion channels such as the nicotinic acetylcholine receptor (4, 5), their size as measured by gel filtration and sucrose density centrifugation (6, 7), and their image under electron microscopy (8). To date, 15 $GABA_A$ subunits have been cloned ($\alpha_{1-6}$, $\beta_{1-4}$, $\gamma_{1-3}$, δ, and ϵ), and expression studies reveal that for the most characterized subtypes at least one α, one β, and one γ-subunit are required to recover a fully functional $GABA_A$ receptor (9). We have investigated the stoichiometry of the most abundant $GABA_A$ receptor isoform in the brain, $\alpha_1\beta_2\gamma_2$, which is responsible for at least some of the therapeutic effects of benzodiazepines, barbiturates, and steroids (10). Receptors modified with a c-Myc epitope on either the $\alpha_1$-, $\beta_2$-, or $\gamma_2$-subunit were expressed transiently in HEK293 cells. It has previously been shown that receptors transiently transfected in these cells using the same methods as employed here are appropriately assembled into αβγ-hetero-oligomers and are expressed on the cell surface. Other subunit assemblies do not reach the cell surface and are retained in the endoplasmic reticulum (12).

mAbs to the c-Myc epitope were derivatized with the fluorescence donor, europium cryptate (EuK), or the fluorescent energy acceptor, XL-665. Europium, encaged by cryptate, emits a strong long lived fluorescent signal at 620 nm when illuminated with light at 337 nm from a nitrogen laser, which can be time-resolved from short lived background fluorescence. In addition to being used as a single label, analogous to a radiolabel for example, EuK also serves as an energy donor. The fluorescent signal produced by EuK can be transferred to an acceptor molecule if it is in close enough proximity. The recipient molecule for this fluorescent resonance energy transfer is a modified allophycocyanine, XL665, which fluoresces at 665 nm (for review of the homogeneous time-resolved fluorescence technology see Mathis (1)). The transfer of energy from europium cryptate-labeled c-Myc antibodies to XL-665-labeled antibodies is therefore indicative of the two antibodies being in close proximity. Because the energy transfer is 50% at a distance of 9.5 nm (1), this would require that the antibodies are very closely associated, for example in the same macromolecular complex.

We used EuK-labeled c-Myc mAbs to quantify the number of subunits present on intact cells expressing $\alpha_1\beta_2\gamma_2$ subunits where each of the subunits was epitope-tagged with c-Myc and compared this with the number of receptors present using conventional radioligand binding. Fluorescence energy transfer was then used to confirm the stoichiometry of the receptor as $(\alpha_1)_2(\beta_2)_2(\gamma_2)_1$.

## EXPERIMENTAL PROCEDURES

[*methyl*-$^3$H]Ro15-1788 (87.0 Ci/mmol) was from NEN Life Science Products, Hertfordshire, United Kingdom. Flunitrazepam, GABA, and fetal calf serum were from Sigma. Minimal essential medium (MEM) was from Life Technologies, Inc.

*Construction of c-Myc Epitope-tagged $GABA_A$ Receptor Subunits and*

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[1] The abbreviations used are: $GABA_A$, γ-aminobutyric acid, type A; GABA, γ-aminobutyric acid; mAb, monoclonal antibody; EuK, europium cryptate; MEM, minimum essential medium; FCS, fetal calf serum.

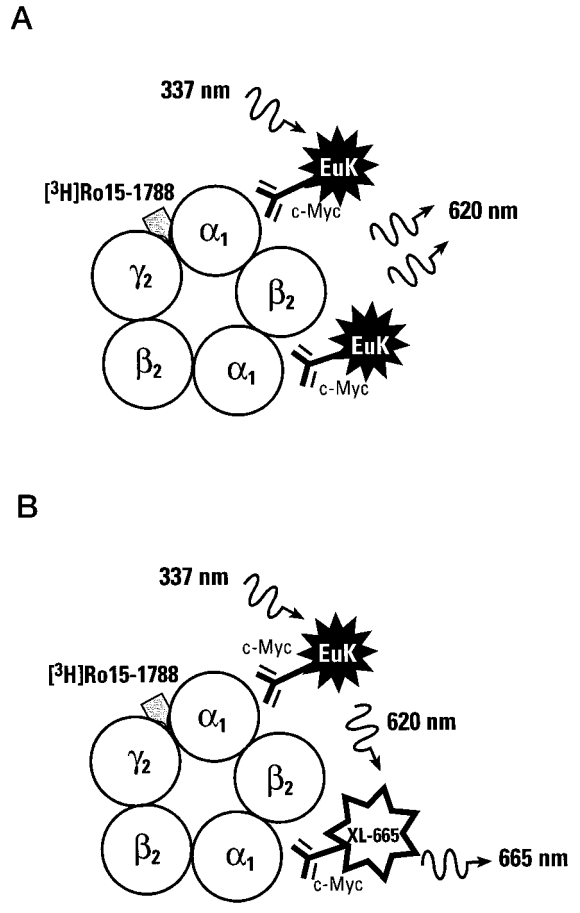


FIG. 1. **Schematic representation of the experimental design.** *A*, the number of subunits present is quantified using saturation binding of EuK-derivatized c-Myc. This is compared with the number of benzodiazepine binding sites using the radioligand [$^3$H]Ro15-1788. *B*, fluorescence resonance energy transfer occurs only when two copies of a subunit are present.

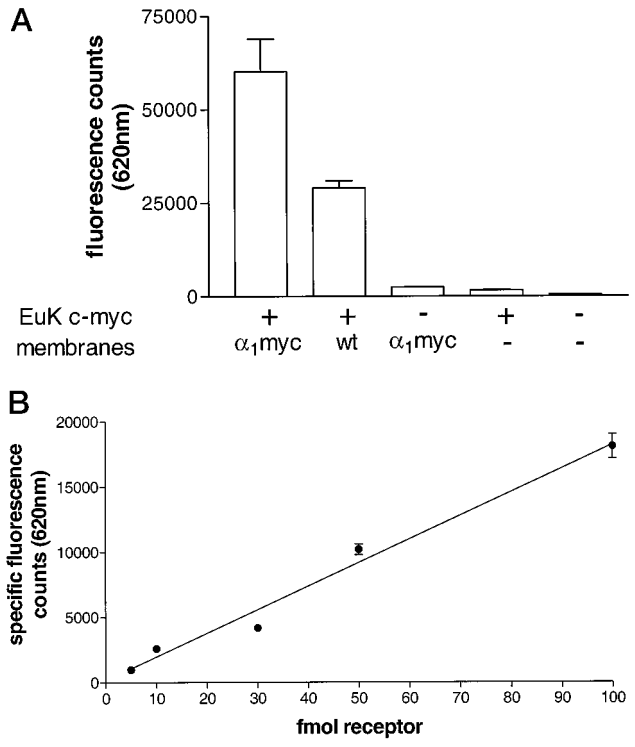


FIG. 2. **The EuK signal from $\alpha_1$(c-Myc)$\beta\gamma_2$ cells is specific and linear with receptor density.** Binding of EuK c-Myc was carried out at 3 nM antibody, and following 1 h of incubation with antibody, cells were washed twice before fluorescence at 620 nm was measured. *A*, binding to cells expressing the $\alpha_1$(c-Myc)$\beta_2\gamma_2$ subtype is shown. Data shown are from two to six experiments with 100 fmol of receptors ([$^3$H]Ro15-1788 binding sites/well). *B*, linear relationship between fluorescence counts and receptor concentration is shown. Data shown are the mean ± S.E. from two to eight experiments.

*Transient Transfections*—The GABA$_A$ receptor $\alpha_1$-subunit was epitope-tagged by site-directed mutagenesis using methods described previously (13). The epitope sequence (EQKLISEEDL) was introduced between Glu$^{34}$ and Leu$^{35}$ (these two residues becoming the first and last amino acid of the epitope tag), just C-terminal to the putative signal peptide cleavage site. The $\beta_2$ and $\gamma_2$ GABA$_A$ receptor subunits were epitope-tagged using a modified version of the pcDNA1Amp eukaryotic expression vector (pcDNA1AmpSignalMyc). This vector was constructed from the $\alpha_1$ c-Myc cDNA described above and contains the 5′-untranslated region of bovine GABA$_A$ $\alpha_1$-subunit (GenBank accession no. X05717), the signal peptide and six amino acids of the mature $\alpha_1$-subunit, and the c-Myc epitope tag sequence (EQKLISEEDL (14)) followed by a small polylinker into which the mature polypeptide of the subunit of interest can be inserted. The human $\beta_2$-subunit c-Myc construct contains amino acids Glu$^{38}$–Asn$^{474}$ of $\beta_2$, and the human $\gamma_2$-subunit c-Myc construct contains amino acids Tyr$^{46}$–le$^{467}$. All constructs were confirmed by DNA sequencing. Constructs were prepared using polymerase chain reaction to generate the appropriate $\beta_2$ and $\gamma_2$ sequences, which were then subcloned into the pcDNA1AmpSignalMyc vector.

DNAs were prepared for transfection by CsCl centrifugation. Transient transfection into HEK293 cells was performed exactly as described previously in detail using a 1:1:1 ratio of $\alpha_1$, $\beta_2$, and $\gamma_2$ cDNAs (12, 15).

*Radioligand Binding*—The benzodiazepine site of the GABA receptor was labeled by the antagonist [$^3$H]Ro15-1788, a radioligand frequently used to quantify receptors, because only fully assembled $\alpha\beta\gamma$ heterotrimers bind this ligand (7, 12, 16). Nonspecific binding was determined using 10 $\mu$M flunitrazepam. Binding to the GABA binding site was carried out with [$^3$H]muscimol, and 100 $\mu$M GABA was used to define nonspecific binding. Radioligand binding assays were carried out in a total volume of 0.5 ml in 10 mM $KH_2PO_4$, 100 mM KCl, pH 7.4, for 1 h at 4 °C prior to termination through Whatman GF/C filters followed by 3× 3-ml washes with cold assay buffer and scintillation counting. In binding studies carried out at one concentration of [$^3$H]Ro15-1788, this was 1.8 nM.

*Immunoprecipitation*—GABA$_A$ receptors solubilized from stably transfected cells were immunoprecipitated using anti-$\alpha_1$-, $\beta$-, or $\gamma_2$-subunit antisera bound to protein A-Sepharose (7). 50 $\mu$l of polyclonal antiserum were incubated with 50 $\mu$l of packed protein A beads in a total volume of 1 ml of Tris-buffered saline for 1 h at room temperature. Receptors were solubilized from the cells using a deoxycholate/Triton buffer (1% Triton X-100, 0.5% deoxycholate, 0.1 mM KCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 100 mM Tris-HCl, pH 8.2) by mixing cell membranes for 1 h at 4 °C with a detergent buffer at a protein concentration of 1 mg/ml.

Aliquots (500 $\mu$l) of detergent-solubilized cell membranes were incubated with antibodies immobilized on protein A beads overnight at 4 °C. The receptor immobilized on protein A beads was washed three times with Tris-buffered saline/0.1% Tween 20 by centrifugation and resuspension and was finally resuspended in 0.5 ml of Tris-buffered saline, and 10–50-$\mu$l aliquots of packed beads were used for [$^3$H]Ro15-1788 binding.

*Generation of Europium Cryptate and XL665-labeled Antiserum*—The monoclonal anti-c-Myc mAb, 9-E10 (ATCC no. CRL-1729, Ref. 14) was used for these experiments. An antibody was purified from hybridoma supernatant by Cymbus Biotech, UK, and this was then derivatized with either the fluorophore, europium cryptate (at an average ratio of 9 molecules of europium cryptate per antibody molecule), or with the fluorescence acceptor, XL665 (at a ratio of 1 molecule of XL-665 per antibody molecule), by Cis-Bio International, Marcoule, France. Labeling of c-Myc mAb with EuK at this ratio gave 418 counts of fluorescence/s/fmol of antibody.

*Binding of Europium Cryptate-labeled mAbs*—Intact cells, transiently transfected with GABA$_A$ receptors with c-Myc-tagged subunits, were harvested by scraping, washed once by centrifugation (20 s at 1,000 rpm in a bench top Eppendorf Microcentrifuge), and resuspended in MEM + 5% FCS. [$^3$H]Ro15-1788 binding (1.8 nM) was carried out to determine receptor density on intact cells. Cells containing the equivalent of 100 fmol of receptor were incubated with various concentrations

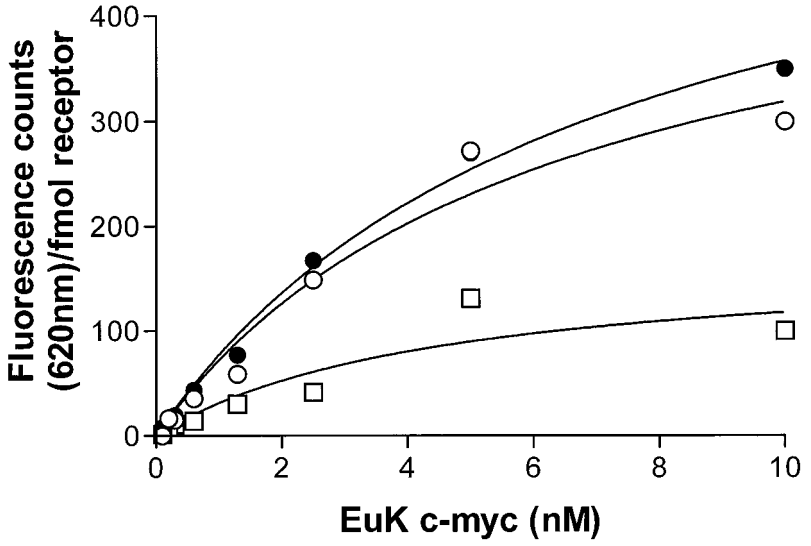


FIG. 3. **Saturation binding of EuK c-Myc to intact cells expressing $\alpha_1$(c-Myc)$\beta_2\gamma_2$, $\alpha_1\beta_2$(c-Myc)$\gamma_2$, and $\alpha_1\beta_2\gamma_2$(c-Myc).** Data shown are specific fluorescence counts bound after subtraction of nonspecific binding to cells expressing $\alpha_1\beta_2\gamma_2$. Data shown are from one experiment that was repeated three times with similar results. Data were fitted using Excelfit, and the binding parameters obtained were: $B_{max}$ = 515 fluorescence counts/fmol of receptors and $K_d$ = 6.2 nM for $\alpha_1$(c-Myc)$\beta_2\gamma_2$ (*open circles*); $B_{max}$ = 602 fluorescence counts/fmol of receptors, $K_d$ = 6.8 nM for $\alpha_1\beta_2$(c-Myc)$\gamma_2$ (*closed circles*); and $B_{max}$ = 169 fluorescence counts/fmol, $K_d$ = 4.4 nM for $\alpha_1\beta_2\gamma_2$(c-Myc) (*open squares*). For calculation of fluorescence counts/fmol of receptors, a receptor was quantified using [$^3$H]Ro15-1788 binding at 1.8 nM.

of europium cryptate-derivatized mAb (0.3–30 nM) in MEM + 5% FCS at room temperature, in a volume of 1 ml for 1 h on a rotamix wheel. After 3× 1-ml washes in MEM + 5% FCS, the cells were resuspended in 190 $\mu$l of MEM + 5% FCS, and the fluorescence signal derived from europium cryptate-labeled c-Myc mAbs was quantified in a 96-well low fluorescence microplate on the Packard discovery fluorescence plate reader, following the addition of 10 $\mu$l of 1 M KF (final concentration, 50 mM KF). In preliminary experiments the inclusion of 5% FCS was found to improve the viability of the cells, and KF was included to stabilize europium cryptate and prevent oxidation of unliganded cryptate.

*Energy Transfer to XL665-labeled Antibodies*—XL665-derivatized mAbs were added in excess (10 nM) to each well, and the plate was read over a 24-h time period. Energy transfer was optimal after the receptor had been incubated with c-Myc-XL665 for 1 h, and the signal was stable for up to 18 h afterward.

## RESULTS

Each of the $\alpha_1$-, $\beta_2$-, and $\gamma_2$-subunits was engineered to express a c-Myc epitope tag at the N terminus, and cells expressing c-Myc-tagged $\alpha$-, $\beta$-, or $\gamma$-subunits or untagged receptors were generated by transient transfection. GABA$_A$ receptors were measured using two ligands, [$^3$H]muscimol, a radioligand for the GABA binding site, and [$^3$H]Ro15-1788, a radioligand for the benzodiazepine binding site, which binds only to fully assembled receptors that contain an $\alpha$-, $\beta$-, and $\gamma$-subunit (7, 9).

*Homogeneity of Expressed Receptors*—In preliminary experiments it was important to confirm that a homogeneous population of fully assembled receptors was being expressed in the cells. This was done in two ways. First the number of binding sites for [$^3$H]muscimol and [$^3$H]Ro15-1788 was compared. Most evidence supports the presence of two [$^3$H]muscimol binding sites and one [$^3$H]Ro15-1788 binding site in a GABA receptor monomer in receptors immunoprecipitated from rat brain (7, 22). Therefore, if the majority of receptors is correctly assembled in cell lines, the ratio of the $B_{max}$ values for [$^3$H]muscimol binding:[$^3$H]Ro15-1788 binding should be 2:1. It is possible that receptors could be expressed that are composed of $\alpha$- and $\beta$-subunits only, but these would not bind [$^3$H]Ro15-1788, and the ratio would therefore be higher. Saturation analysis of [$^3$H]muscimol and [$^3$H]Ro15-1788 binding was carried out in cells expressing the untagged $\alpha_1$-, $\beta_2$-, and $\gamma_2$-subunits. Maximal binding of [$^3$H]muscimol was 2067 ± 98 fmol/mg of protein with a $K_d$ of 5.6 ± 0.7 nM ($n$ = 3). Maximal binding of [$^3$H]Ro15-1788 was 1226 ± 21 fmol/mg of protein with a $K_d$ of 0.7 ± 0.05 nM ($n$ = 3). The ratio of the GABA:benzodiazepine sites was 1.74 ± 0.11, which is in agreement with the expression of $\alpha\beta\gamma$ heterotrimeric receptors and no significant expression of receptors that contain only $\alpha\beta$-subunits.

Second, the ability of antibodies raised against the $\alpha_1$-, $\beta_2$-, and $\gamma_2$-subunit to immunoprecipitate all the [$^3$H]Ro15-1788 binding sites from a solubilized cell membrane preparation was compared. The majority of receptors could be immunoprecipitated with antibodies raised against the $\alpha_1$-, $\beta$-, or $\gamma_2$-subunits ($\alpha_1$, 74 ± 3%; $\beta$, 81 ± 7%; and $\gamma_2$, 90 ± 4% of [$^3$H]Ro15-1788 binding sites, $n$ = 3). This confirms that the receptors that bind [$^3$H]Ro15-1788 contain at least one $\alpha$-, one $\beta$-, and one $\gamma$-subunit.

*Expression of c-Myc-tagged GABA$_A$ Receptors*—The number of c-Myc-tagged subunits was quantified by labeling with anti-c-Myc mAb derivatized with europium cryptate, as illustrated in Fig. 1 (using the subunit arrangement proposed by Tretter *et al.* (11) as a model).

The presence of a c-Myc tag on the N terminus of the GABA$_A$ receptor subunits did not affect the expression of the receptor. The number of binding sites present on cells was unaffected by the addition of the epitope tag, and saturation analysis of [$^3$H]Ro15-1788 binding showed that there was no major difference in the affinity of receptors for ligand ($K_d$ is as follows: $\alpha_1\beta_2\gamma_2$ = 1.3 nM; $\alpha_1$(c-Myc)$\beta_2\gamma_2$ = 1.1 nM; $\alpha_1\beta_2$(c-Myc)$\gamma_2$ = 1.4 nM; $\alpha_1\beta_2\gamma_2$(c-Myc) = 1.3 nM, $n$ = 1). The density of receptors was unaffected by the expression of subunits with a c-Myc epitope tag, being 1.2–3.1 pmol/mg of protein. This is in agreement with previous studies where GABA$_A$ receptors were tagged with c-Myc and FLAG epitopes without compromising binding, function, or modulation of the GABA$_A$ receptor (12).

*Labeling of GABA$_A$ Receptors with EuK-c-Myc Monoclonal Antibodies*—Preliminary experiments were carried out to determine the optimal conditions for the binding of EuK-labeled c-Myc mAbs. As shown in Fig. 2*B*, the fluorescence signal emitted by EuK c-Myc at 620 nm was linear with receptor concentration. Therefore the binding of EuK-labeled c-Myc to the receptor was linear up to at least 100 fmol of receptor/ml. Background fluorescence from the plate and other reagents

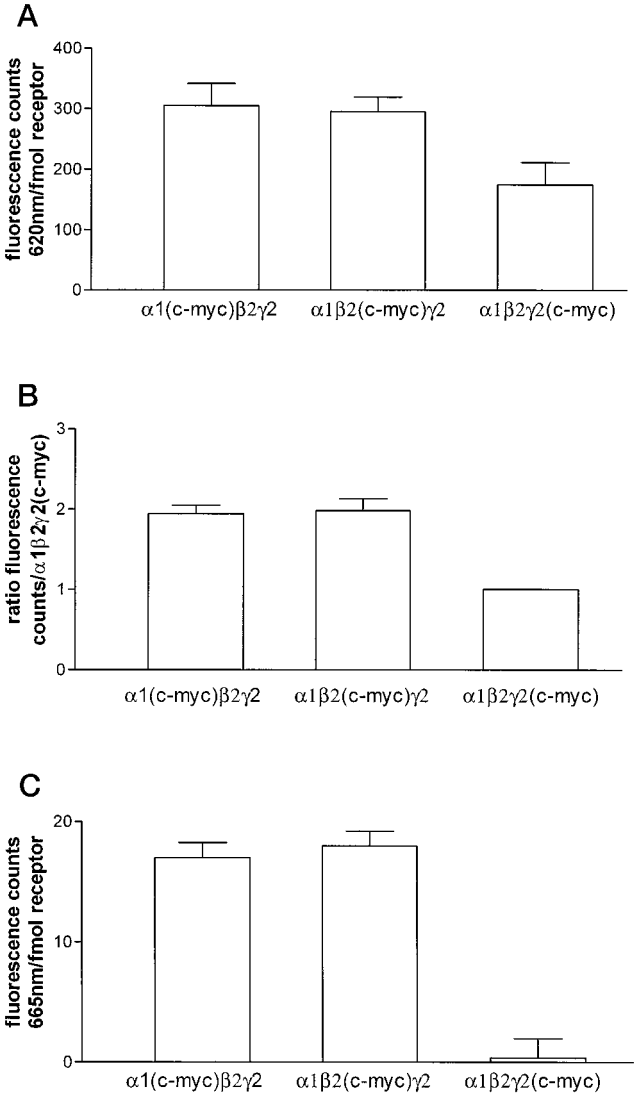


FIG. 4. **Fluorescence signal observed at 620 and 665 nm from cells expressing $\alpha_1$(c-Myc)$\beta_2\gamma_2$, $\alpha_1\beta_2$(c-Myc)$\gamma_2$, and $\alpha_1\beta_2\gamma_2$(c-Myc).** Data shown are the mean ± S.E. of six independent experiments. The specific fluorescence counts observed at 620 nm for $\alpha_1$(c-Myc)$\beta_2\gamma_2$ were 315 ± 37/fmol and for $\alpha_1\beta_2$(c-Myc)$\gamma_2$ were 286 ± 41/fmol. These were both significantly higher than that observed with $\alpha_1\beta_2\gamma_2$(c-Myc), 156 ± 21/fmol. The mean ± S.E. and the ratio for binding relative to $\alpha_1\beta_2\gamma_2$(c-Myc) were calculated for each experiment and are shown in *panel B*. The ratios of binding for $\alpha_1$(c-Myc) $\beta_2\gamma_2$:$\alpha_1\beta_2\gamma_2$(c-Myc) and $\alpha_1\beta_2$(c-Myc)$\gamma_2$:$\alpha_1\beta_2\gamma_2$(c-Myc) were not significantly different from 2.0 ($p < 0.05$, Student's *t* test). *Panel C,* in the fluorescence energy transfer experiments, where emission was measured at 665 nm, there was no significant difference between the fluorescence counts produced by $\alpha_1$(c-Myc)$\beta_2\gamma_2$ (16.7 ± 1.5) and $\alpha_1\beta_2$(c-Myc)$\gamma_2$ (17.8 ± 1.4), but both were significantly more than $\alpha_1\beta\gamma_2$(c-Myc) (0.38 ± 2.4), which was not significantly more than 0.

accounted for the fluorescence in the absence of added EuK, and this was typically 800–1500 counts/s as shown in Fig. 2*A*. Binding of EuK c-Myc to cells expressing untagged receptors was significant (Fig. 2*A*). Efforts were made to try to reduce this nonreceptor-mediated binding by extensive washing following incubation with EuK-c-Myc mAb and preincubation of cells with IgG to block any IgG binding sites on the cells. Neither of these measures reduced nonspecific binding to control cells by more than 10% and were not routinely adopted. Instead, each experiment contained a wild type control (*i.e.* receptors expressed without any c-Myc epitope present), and nonspecific binding to these cells was subtracted from the total fluorescence signal, which was analogous to the methodology employed for defining nonspecific binding in a radioligand binding assay.

*Saturation Binding of EuK-c-Myc Antibodies to $\alpha_1$(c-Myc)$\beta_2\gamma_2$, $\alpha_1\beta_2$(c-Myc)$\gamma_2$, and $\alpha_1\beta_2\gamma_2$(c-Myc)*—Saturation binding experiments were carried out using control cells expressing $\alpha_1\beta_2\gamma_2$ and cells expressing receptors where either the $\alpha$-, $\beta$-, or $\gamma$-subunit was epitope-tagged with c-Myc. Nonspecific binding of EuK c-Myc to control cells expressing an untagged receptor was subtracted from total binding at each concentration of antibody and was routinely 50% at 3 nM antibody. In all cases binding was saturable and had a high affinity, and EuK-labeled c-Myc bound to more sites in the $\alpha_1$(c-Myc)$\beta_2\gamma_2$ and $\alpha_1\beta_2$(c-Myc)$\gamma_2$ compared with $\alpha_1\beta_2\gamma_2$(c-Myc) as exemplified in Fig. 3. To quantitate this, the experiment was repeated on six separate occasions using 3 nM antibody, and the specific fluorescence counts are shown in Fig. 4*A*. The ratio of fluorescence labeling to $\alpha_1$(c-Myc)$\beta_2\gamma_2$, $\alpha_1\beta_2$(c-Myc)$\gamma_2$, and $\alpha_1\beta_2\gamma_2$(c-Myc) was compared as shown in Fig. 4*B*. This clearly shows that there are twice as many EuK c-Myc sites on receptors where the $\alpha$- or $\beta$-subunit is tagged compared with receptors where the $\gamma$-subunit is tagged (ratio of $\alpha_1$(c-Myc)$\beta_2\gamma_2$:$\alpha_1\beta_2$(c-Myc)$\gamma_2$:$\alpha_1\beta_2\gamma_2$(c-Myc) = 1.89 ± 0.12:1.94 ± 0.18:1).

A comparison of the $B_{max}$ values for [$^3$H]Ro15-1788 and EuK-c-Myc fluorescence can be used to calculate the number of binding sites for the antibody/benzodiazepine binding site. For $\alpha_1$(c-Myc)$\beta_2\gamma_2$ there are 315 ± 37 fluorescence counts/fmol of Ro15-1788 binding sites using 3 nM antibody and 1.8 nM [$^3$H]Ro15-1788 (Fig. 4). Using Clarke's equation (occupancy = [ligand]/[ligand] + $K_i$) this is equivalent to 610 ± 71 fluorescence counts/fmol of Ro15-1788 binding sites. Given that there are 418 fluorescence counts of EuK/fmol of antibody (see "Experimental Procedures"), there are calculated to be 1.46 ± 0.16 antibody binding sites/[$^3$H]Ro15-1788 binding site. The limitations and multiple sources of error in this experiment (*e.g.* aggregation of antibody, heterogeneity of antibody labeling, etc.) would be more likely to lead to an underestimate of the number of antibody binding sites therefore the observation that there is more than one antibody binding site/[$^3$H]Ro15-1788 binding site supports the most likely stoichiometry of 2 $\alpha$-subunits/receptor:1 benzodiazepine site.

*Fluorescence Energy Transfer Studies*—The affinity of the EuK-derivatized c-Myc antibodies for the GABA$_A$ subunits was 4–6 nM as shown in Fig. 3. A concentration of 3 nM was selected for the fluorescence transfer studies because this would less than half-maximally saturate the receptor. Following incubation with 3 nM EuK-derivatized c-Myc mAb, cells were washed and incubated with excess XL665-derivatized c-Myc mAb, and fluorescence at 665 nm was measured in response to laser excitation. As shown in Fig. 4*C*, a specific signal is observed, indicating energy transfer when either the $\alpha_1$- or the $\beta_2$-subunit is epitope-tagged (16.7 ± 1.5 counts/fmol and 17.8 ± 1.4 counts/fmol) but not when the $\gamma_2$-subunit is epitope-tagged. Because energy transfer can only take place when there are two c-Myc epitopes in close proximity, *i.e.* two subunits in the same receptor complex, this confirms that the stoichiometry of the GABA$_A$ receptor under study is $(\alpha_1)_2(\beta_2)_2(\gamma_2)_1$. Furthermore, the lack of any observable signal with $\alpha_1\beta_2\gamma_2$(c-Myc) could be interpreted as evidence that receptors are not primarily expressed as clusters on the cell surface. If this were the case, it may be possible to observe energy transfer between $\gamma_2$-subunits on separate receptors.

## DISCUSSION

To date, the stoichiometry of the GABA$_A$ receptor has been approached both directly and indirectly. Many laboratories have observed that a receptor can contain two different types of $\alpha$-subunits (16–20). Conflicting evidence has been obtained on

whether two types of γ-subunit can coexist in a single receptor monomer with two studies proposing that two γ-subunits can be present (21, 22). There have been fewer studies on the presence of two β-subunits because the structural similarity of the β-subunits has precluded the development of antibodies, which clearly distinguish between them.

There have been four studies that directly analyzed the stoichiometry of the receptor. The first two, chronologically, have taken an electrophysiological approach. Backus *et al.* (23) favor the composition 2× α, 1× β, and 2× γ based on measuring the outward current induced by point mutation of charged amino acids on either side of the TM2 domain. Chang *et al.* (24) used a similar method (mutation of a lysine in TM2) to increase the sensitivity of the receptor to GABA in proportion to the number of mutant subunits present. In contrast to Backus *et al.* (23), they conclude that the stoichiometry is 2× α, 2× β, and 1× γ, as do Im *et al.* (25) from studies of tandem subunit constructs. The most recent study used antibody labeling of chimeric subunits to determine the ratio of subunits present from Western blots, and they conclude that the structure is also 2× α, 2× β, and 1× γ (11).

The approach taken here has used fluorescently derivatized mAbs to quantify subunits relative to the benzodiazepine binding site on the receptor and fluorescent resonance energy transfer to confirm that the stoichiometry of the receptor is $(\alpha_1)_2(\beta_2)_2\gamma_2$. This method has several advantages over other previously described methods. 1) It uses intact cells; therefore only receptors that are expressed on the surface (and are therefore presumed to be correctly assembled) are considered (12, 26). 2) It involves minimal disruption of receptor structure by requiring only epitope tagging, which does not affect the expression or function of the receptor (12). 3) Only small amounts of transiently transfected material are required.

Furthermore this approach has general applicability to other multisubunit cell surface proteins and can be used particularly to investigate the stoichiometry of subunits in GABA$_A$ receptors composed of rarer subunits and in other ligand-gated ion channels.

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